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PRINCIPAL INVESTIGATOR: Yipin Lu

CONTRACTING ORGANIZATION: The University Michigan Medical School
Ann Arbor, Michigan 48109-0602

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Yipin Lu

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The University Michigan Medical School
Ann Arbor, Michigan 48109-0602

E-Mail: yipinl@umich.edu

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The inhibitors of apoptosis protein (IAP) are intrinsic cellular negative regulators of apoptosis. The X-linked inhibitor of apoptosis protein (XIAP) is a potent caspase inhibitor in IAP family, which is highly expressed in most of the widely studied breast cancer cell lines. The mitochondrial protein Smac is a negative regulators of XIAP that competitively binds to a binding pocket on the BIR3 domain of XIAP and disrupts caspase-9 binding to XIAP. Using the 3D structure of XIAP, we have performed a structure-based database screening of large chemical databases and discovered several non-peptide small molecule inhibitors of XIAP. The most potent compound among them, SMXI-56, binds to the XIAP BIR3 protein with an affinity similar to that of the natural Smac peptide in a fluorescence polarization-based binding assay. The NMR HMQC analysis confirmed that SMXI-56 interacts with several crucial residues in the XIAP BIR3 domain where Smac and caspsase-9 bind. SMXI-56 inhibits cell growth and induces apoptosis in breast cancer cells with high levels of XIAP, but has a minimal effect on normal breast cells with low levels of XIAP. This work demonstrates that the virtual database screening combined with biological activity tests can identify potential inhibitors of XIAP for treatment of breast cancer.

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Introduction:

The inhibitor of apoptosis proteins (IAPs) represents an important class of negative regulators of programmed cell death which block cell death by inhibition of distinct caspases. XIAP (X-linked IAP) is an important member of IAP family of proteins. XIAP regulates apoptosis by binding to and inhibiting caspase-3, -7, and -9. Consistent with its role as a major negative regulator of apoptosis, XIAP is found to be widely expressed in human cancers. We have found that several breast cancer cell lines (MDA-MB-231, MCF-7, MDA-MB-435) have high levels of XIAP expression. XIAP overexpression may be an important factor leading to apoptotic resistance in human breast cancer. The mitochondrial protein Smac/DIABLO is a negative regulators of XIAP. Smac competitively binds to a surface binding pocket on the BIR3 domain of XIAP and disrupts caspase-9 binding to XIAP, which is critical for Smac's inhibition of apoptosis. The 3D structures of BIR3 domain of XIAP in complex with Smac protein/peptide show that the interaction between Smac and XIAP is mediated by only four Smac residues and a small but well-defined binding groove in the BIR3 domain of XIAP, which is suitable for designing small molecular inhibitor of XIAP.

In this grant, we propose an effective structure-based approach to discover small non-peptide molecules that bind to the surface pocket of the BIR3 domain of XIAP where Smac binds. These non-peptide, drug-like small molecules that bind to XIAP may block the anti-apoptotic function of XIAP, which in turn may induce apoptosis in human breast cells with a high level of XIAP expression. Hence, designing of small molecule inhibitors targeting XIAP at this BIR3 binding site may represents an attractive approach for the development of a novel therapy for the treatment of breast cancer with XIAP overexpression. We propose to use a powerful structure-based 3D-database screening approach to discover small organic molecules that bind to the BIR3 binding groove of XIAP. The binding affinities of these potential XIAP inhibitors to the BIR3 domain of XIAP will be determined by the fluorescence polarization-based method followed by conclusive confirmation by the NMR-based methods. We will also investigate their activity in inhibition of breast cell growth and induction of apoptosis in breast cancer cells, their action specificity, and their molecular mechanism of action.

The success of this project will pave the way for the development of a small molecule drug through modulation of the anti-apoptotic function of XIAP protein for the treatment of breast cancer with XIAP overexpression. Such a novel target-specific anticancer therapy will be particularly effective when used in combination with conventional chemotherapy or radiation therapy for the treatment of breast cancer by overcoming drug resistance of breast cancer cells.

Body of the report:

Task 1. Structure-based database searching and computational docking (**1-18 months**).

During the past 12 months, we have completed the 3D-database searching of three 3D-database: the National Cancer Institute's 3D-database of more than 250,000 synthetic organic compounds and natural products, the Merck Index database of more than 6000 chemical substances including human drugs and natural products, and an in-house Herbal Medicine database of more than 8000 small organic molecules isolated from herbal medicines. We used a hybrid pharmacophore-based and structure-based database screening method. At the first stage, a pharmacohpore model was developed based upon the structures of known XIAP inhibitors and was used to perform a pharmacophore search of the databases to identify potential XIAP inhibitors. Chemical structural analysis on known XIAP peptide mimetic inhibitors indicated that a hydrogen bond donor at the N-terminal and a hydrophobic group at the C-terminal of the inhibitor are important for the binding. Accordingly, a pharmacophore model was proposed, as shown in Figure 1. Using this pharmacophore model, we searched the databases with the program Catalyst Version 4.9 (Accelrys, SanDiego, CA). A total of 39520 compounds were identified as hits, which satisfy the chemical and geometrical requirements specified in the pharmacophore model (Figure 1). By using pharmacophore searching as the first filter, we significantly reduced the number of compounds for computational structure-based screening, which enable us to use the computational docking methods that are more accurate but may be less fast. In the second stage, the hits identified from the pharmcophore searching were further screened through structure-based searching using docking program GOLD version 2.1 (The Cambridge Crystallographic Data Centre, Cambridge, UK). A consensus score method was employed to re-evaluate the hits from the structure-based search by scoring functions Xscore and Drugscore. The top 500 scored compounds common to each scoring function were identified as candidates for bioassay.

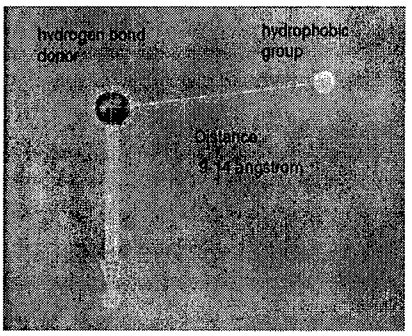


Figure 1. A pharmacophore model derived from the peptide mimetic inhibitors of XIAP.

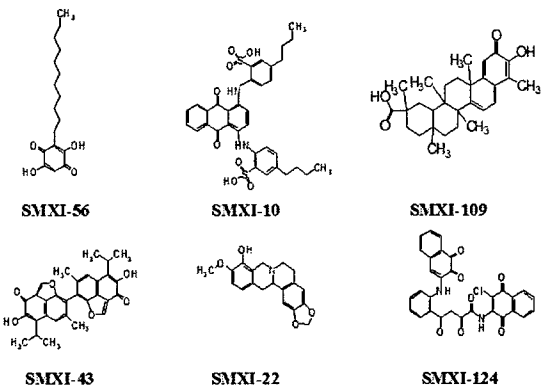
Task 2. *In vitro* biological confirmation of potential XIAP inhibitors and mechanism investigations (**3-36 months**).

Task 2.1. Screen candidate small molecule inhibitors using the established *in vitro* fluorescence polarization (FP) based binding assay and confirm the binding to XIAP BIR3 domain by NMR experiments.

Our group has established a sensitive and quantitative in vitro binding assay using fluorescence polarization (FP) based method, which is a solution-based assay and is highly suitable for high-throughput screening. The interactions between XIAP-BIR3 and Smac peptides form the basis for development of the FP-based assay. A fluorescent labeled mutated 7-mer Smac peptide **SM7F** (ARPFAQK, Kd = 0.061 μM), and the human XIAP-BIR3 protein (residue 241-356) with an His tag were used in our competitive binding assay. We have chosen to use 10

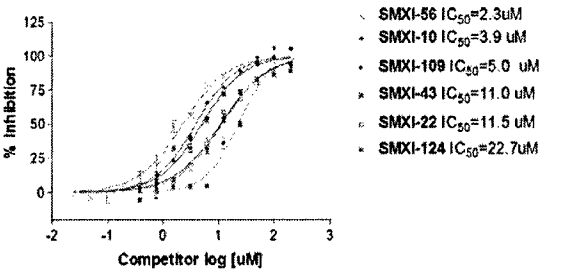
nM SM7F and 0.060 μM XIAP-BIR3 protein as the assay conditions based on several criteria: 0.060 μM XIAP is close to the actual Kd of SM7F and the tracer is saturated about 50%, making the assay very sensitive; 10 nM of SM7F has sufficient fluorescence intensity to overcome of the fluorescence background for some inhibitors. Under these conditions, the assay range (mP of bound peptide – mP of free peptide) is 95.2 ± 3.0 mP which is a large polarization signal window, allowing sensitive detection of a small decrease in polarization.

Figure 2. chemical structures of 6 most potent small molecule inhibitorsof XIAP



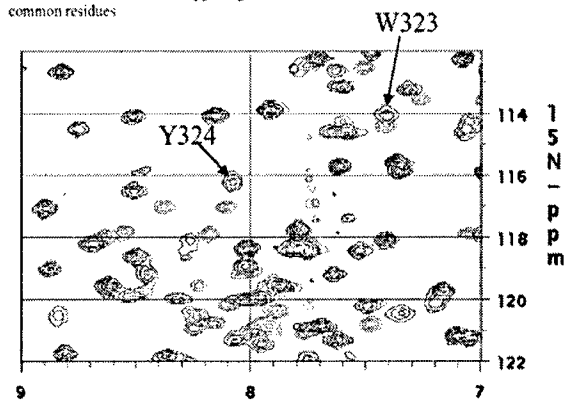
Using this method, we screened over 200 candidate small molecule inhibitors to date, initially at two doses of 200 and 100 μM. Forty-one compounds showed inhibitory activity of more than 50% at 200 μM and were classified as active. Twenty compounds displayed more than 90% inhibition at 100 μM and further dose-dependent binding experiments were carried out to determine their IC50 values. Of which, four compounds were found to have high fluorescence and were excluded from further evaluation in the binding assay. All other 16 active compounds displayed a dose-dependent inhibition of Smac peptide binding to XIAP. The chemical structures of these six most potent compounds are shown in Figure 2. Figure 3 presents the binding curves of the 6 most potent compounds.

Figure 3. Competitive binding curves of 6 non-peptide small molecule inhibitors and their IC_{50} values.



SMXI-56 (denoted as Small Molecule XIAP Inhibitor) is the most potent inhibitor among these 170 compounds in the binding assay (Figure 3). SMXI-56 (IC_{50} = 2.3 μM) is as potent as the natural Smac peptide (IC_{50} = 3.1 μM). The Hill slope analysis showed that SMXI-56 has a single binding site and no deviation from the ideal model (Hill slope = 1). SMXI-56 is a natural product (embelin) which doesn't have fluorescence by itself.

Figure 4. Superposition of ^{15}N-HSQC spectra of free XIAP BIR3 domain (black) and that of the XIAP BIR3 with SMXI-56 (red). W323 and Y324 were found to be affected by SMXI-56, similar to Smac, suggesting that SMXI-56 and Smac both interact with these common residues



Two other compounds SMXI-10 and SMXI-109 have IC_{50} values, similar to that of the natural Smac peptide. Three other compounds have IC_{50} values between 11 and 23 μM. Of note, all these compounds

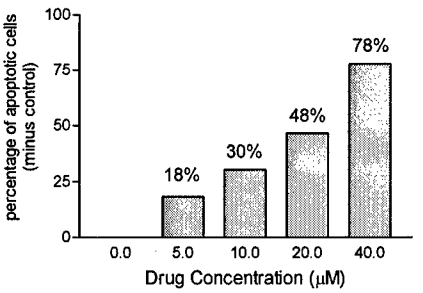
are non-peptides and belong to different chemical classes. Therefore, our structure-based searching has led to the discovery of several non-peptide small molecule inhibitors of XIAP.

To conclusively confirm that SMXI-56 binds to the XIAP BIR3 domain where Smac binds, we performed an analysis using nuclear magnetic resonance (NMR) methods. ^{15}N Heteronuclear Single Quantum Coherence Spectroscopy (HSQC) NMR spectra were recorded with samples containing ^{15}N XIAP protein with SMXI-56 or without SMXI-56. Overlay of two ^{15}N HSQC spectra of the BIR3 domain of human XIAP with SMXI-56 (red) and without (black) is shown in Figure 4. It was found that several residues were affected by the binding of SMXI-56, including Trp 323 and Tyr 324 residues, which are two crucial residues when Smac binds of XIAP according to the crystallographic structures of XIAP in complex with Smac. Thus, our NMR studies conclusively show that SMXI-56 binds to the binding pocket in the XIAP BIR3 domain where Smac binds.

Task 2.2. Induction of apoptosis by small molecule inhibitor

We have tested the ability of SMXI-56 in induction of apoptosis in MDA-MB-231 breast cancer cells using the TACS^{TM} Annexin V-FITC Kit. MDA-MB-231 has previously been shown to have a high level of XIAP protein and we have confirmed this analysis through our own Western blotting analysis (data not shown). SMXI-56 induces apoptosis in a dose-dependent manner (Figure 5). At 5 μM, 18% of cancer cells underwent apoptosis. At 10, 20 and 40 μM, 30%, 48% and 78% of cancer cells underwent apoptosis (minus control), respectively. SMXI-56 is thus cell-permeable and induces a dose-dependent apoptosis in MDA-MB-231 breast cancer cells.

Figure 5. Induction of apotosis in MDA-MB-231 breast cancer cells by **SMXI-56** at 24 hours (Annexin-V assay, minus control).

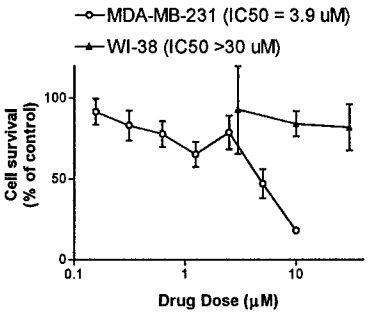


Task 2.3. Inhibition of cell growth by small molecule inhibitors of XIAP

We further evaluate if SMXI-56 may inhibit cell growth in cancer cells with a high level of XIAP. The ability of SMXI-56 to inhibit cell growth in MDA-MB-231 breast cancer cells was test. SMXI-56 potently inhibits cell growth with an IC_{50} value of 3.9 μM in a MTT assay (Figure 6).

To test the specificity of SMXI-56, we test its activity in normal fibroblast WI-38 cell line with an undetectable level of XIAP (data not shown). SMXI-56 has less than 20% of inhibition in cell growth at 30 μM in WI-38 cell line (Figure 6), thus displaying specificity to normal fibroblast WI-38 cells.

Figure 6. Inhibition of cell growth by SMXI-56 in human breast cancer MDA-MB-231 and fibroblast WI-38 cell lines.



Key Research Accomplishments:

(1). we have discovered several non-peptide small molecule inhibitors of XIAP by a hybrid pharmacophore-based and structure-based 3D-database screening strategy. Three compounds have a similar binding affinity to the natural Smac peptide and protein. SMXI-56 (IC_{50} = 2.3 μM) is as potent as the natural Smac peptide (IC_{50} = 3.1 μM).

(2). We showed that SMXI-56 inhibits cell growth in MDA-MB-231 human breast cancer cell line with an IC_{50} value of 3.9 μM and has a minimal effect on WI-38 fibroblast cell line.

(3). We showed that SMXI-56 potently induces apoptosis in MDA-MB-231 cells in a dose-dependent manner.

Reportable Outcomes:

1. Discovery of Small Molecule Inhibitors of XIAP as Potential Novel Therapy for the Breast Cancer.. Yipin Lu, Zaneta Nikolovska-Coleska, Liang Xu, Zengjian Hu, York Tomita, Dajun Yang And Shaomeng Wang, The fourth Era of Hope meeting, Philadelphia, Pennsylvania, June 8-11, 2005 (accepted)

Conclusions:

Using a powerful hybrid pharmacophore-based and structure-based 3D-database screening strategy, we have discovered and confirmed several small molecule inhibitors that bind to XIAP. Three compounds have a similar binding affinity to the natural Smac peptide and protein. SMXI-56 is as potent as the natural Smac peptide. Using NMR methods, we conclusively confirm that SMXI-56 binds to the binding site in the XIAP BIR3 where Smac binds. SMXI-56 inhibits cell growth in MDA-MB-231 human breast cancer cell line and has a minimal effect on WI-38 fibroblast cell line. SMXI-56 potently induces apoptosis in MDA-MB-231 cells in a dose-dependent manner. Thus, SMXI-56 is a fairly potent cell-permeable small molecule inhibitor of XIAP and represents a promising lead compound for designing an entirely new class of anti-breast cancer agents that target the XIAP. Our work demonstrates that the virtual database screening combined with biological activity tests can identify potential inhibitors of XIAP for treatment of breast cancer.